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Supplemental Assay Method for Isolation of Parainfluenza-3 Virus from Nasal Excretions

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* assay method using Madin-Darby bovine kidney (MDBK) cells for the isolation of parainfluenza 3 virus (PI_3V) from nasal excretions of calves. PI_3V isolation is performed for immunogenicity studies on PI_3 live virus vaccines in accordance with the Code of Federal Regulations, Title 9 (9 CFR).

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Incubator, 36°± 2°C, 5% ± 1% CO_2, high humidity

2.1.2 Water bath, 36º ± 2ºC

2.1.3 Pipettor, 200-µL and 1000-µL, and tips

2.1.4 Vortex mixer

2.1.5 Pipettor, 50- to 300-µL x 12-channel

2.1.6 Microscope, inverted light

2.1.7 Centrifuge and rotor

2.1.8 Pipette-aid

2.2 Reagent/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 PI_3V Positive Control

2.2.2 MDBK cell line found to be free of extraneous agents as tested by the 9 CFR.

2.2.3 Diluent Medium (National Veterinary Services Laboratories [NVSL] Medium #20030)

1. 9.61 g minimum essential medium (MEM) with Earles salts without bicarbonate

2. 2.2 g sodium bicarbonate ($NaHC0_3$)

3. Dissolve with 900 mL deionized water (DI).

4. Add 5.0 g lactalbumin hydrolysate or edamine to 10 mL DI. Heat to 60°± 2°C until dissolved and add to **Section 2.2.3(3)** with constant mixing.

5. Q.S. to 1000 mL with DI; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).

6. Sterilize through a 0.22-µm filter.

7. Aseptically add:

a. 10 mL L-glutamine
b. 50 µg/mL gentamicin sulfate

8. Store at 4°± 2°C.

2.2.4 Sample Transport Medium

1. 200 mL Diluent Medium

2. 30 µg/mL gentamicin sulfate

3. 1.5 µg/mL amphotericin B

4. Store at 4°± 2°C.

2.2.5 Growth Medium

1. 900 mL of Diluent Medium

2. Aseptically add 100 mL gamma-irradiated fetal bovine serum (FBS).

3. Store at 4°± 2°C.

2.2.6 Maintenance Medium

1. 980 mL Diluent Medium

2. Aseptically add 20 mL gamma-irradiated FBS.

3. Store at 4°± 2°C.

2.2.7 Alsevers Solution

1. 20.5 g dextrose ($C_6H_{12}O_6$)

2. 8.0 g sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)

3. 4.2 g sodium chloride (NaCl)

4. 0.55 g citric acid ($C_6H_8O_7 \cdot H_2O$)

5. Dissolve with 100 mL DI.

6. Filter through a 0.22-µm filter.

7. Store at 4°± 2°C.

2.2.8 10X Phosphate buffered saline (10X PBS)

1. 80.0 g sodium chloride

2. 2.0 g potassium chloride (KCl)

3. 2.0 g potassium phosphate, monobasic, anhydrous (KH_2PO_4)

4. Dissolve with 900 mL DI.

5. Add 11.5 g sodium phosphate dibasic, anhydrous (Na_2HPO_4) to 50 mL DI, heat to 60°± 2°C until dissolved. Add to **Section 2.2.8(4)** with constant mixing.

6. Q.S. to 1000 mL with DI.

7. Autoclave at 15 psi, 121°± 2°C for 35 ± 5 minutes.

8. Store at 4°± 2°C.

2.2.9 1X PBS (PBS)

1. 100 mL 10X PBS

2. 900 mL DI

3. Adjust the pH to 7.0-7.3 with 5N sodium hydroxide (NaOH).

4. Store at 4°± 2°C.

2.2.10 Guinea pig red blood cells (RBCs) in an equal volume of Alsevers Solution

2.2.11 7.5 % Sodium Bicarbonate

1. 7.5 g sodium bicarbonate

2. Q.S. to 100 mL DI.

3. Autoclave at 15 psi, 121°± 2°C for 30 ± 5 minutes.

4. Store at 4°± 2°C.

2.2.12 Trypsin Versene (TV)

1. 8.0 g sodium chloride

2. 0.40 g potassium chloride

3. 0.58 g sodium bicarbonate

4. 0.50 g irradiated trypsin

5. 0.20 g versene or disodium salt ethylenediaminetetraacetic acid (EDTA)

6. 1.0 g dextrose

7. 0.4 mL 0.5% phenol red

8. Dissolve with 1000 mL DI.

9. Adjust pH to 7.3 with 7.5% Sodium Bicarbonate.

10. Filter through a 0.22-µm filter.

11. Aseptically dispense into 100 mL amounts and store at -20°± 4°C.

2.2.13 Tissue culture plates, 96-well

2.2.14 Polystyrene tubes, 12 x 75-mm and 17 x 100-mm

2.2.15 Conical tube, 50-mL

2.2.16 Serological pipettes, 10-mL

2.2.17 Cotton-tipped applicators

2.2.18 Reagent reservoir

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training in cell culture techniques, virus isolation, the principles of aseptic technique, and animal care and handling techniques.

3.2 Preparation of equipment/instrumentation

On the day of test initiation, set a water bath at 36°± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 Sample Transport Tube.

Prior to Nasal Excretion Sample collection, fill an appropriate number of 12 x 75-mm polystyrene tubes with 3 mL of sample transport medium for each calf on test. A minimum of 1 tube per calf per day of anticipated collection is prepared.

3.3.2 Nasal Excretion Sample collections

Take Nasal Excretion Samples from each calf by inserting a sterile cotton-tipped applicator several inches into each nasal passage. Immediately immerse the two applicators laden with nasal excretion material into a Sample Transport Tube; labeled appropriately with calf identifier and date of collection. Freeze and store the Sample Transport Tube at -70°± 5°C until virus isolation test is conducted.

3.3.3 Preparation of MDBK cell culture plate (Test Plate)

Cells are prepared from healthy, confluent MDBK cells that are maintained by passing weekly. Two days prior to test initiation and 2 days prior to the 2nd passage, seed 200 µL/well of $10^{5.4}$ to $10^{5.6}$ cells/mL, suspended in Growth Medium into all wells of a 96-well cell culture plate with a multichannel pipettor. At least 2 Nasal Excretion Samples are tested on 1 plate. Cells are removed from the growth containers by using TV solution. The Test Plate is incubated at 36°± 2°C in a CO_2 incubator for 36 ± 12 hours. Cells should be 80% confluent prior to use.

3.3.4 Preparation of PI_3V Positive Control

1. On the day of test initiation, rapidly thaw a vial of PI_3V Positive Control in a 36°± 2°C water bath and make tenfold dilutions.

a. Using a 10-mL serological pipette, dispense 4.5 mL of Dilution Medium in an appropriate number of 17 x 100-mm polystyrene tubes to bracket the expected titer endpoint according to the CVB Reference and Reagent sheet. Label tubes (for example: 7 tubes, labeled 10^{-1} to 10^{-7} respectively).

b. Using a 500 µL pipettor, transfer 500 µL of PI_3V Positive Control to the first tube labeled 10^{-1}; mix by vortexing.

c. Using a new pipette tip, transfer 500 µL from the 10^{-1} labeled tube (**Section 3.3.4[1.b]**) to the 10^{-2} tube; mix by vortexing.

d. Repeat **Section 3.3.4(1.c)** for each of the subsequent dilutions, transferring 500 µL of the previous dilution to the next dilution tube, until the tenfold dilution series is completed.

3.3.5 Preparation of 0.5% RBCs Suspension for the hemadsorption (HAd) test

1. Transfer RBCs to a 50-mL conical tube.

2. Q.S. to 50 mL with Alsevers Solution; mix by inverting several times.

3. Centrifuge at 400 x g for 15 ± 5 minutes (1500 rpm in a J6B centrifuge with a JS-4.0 rotor).

4. Remove supernatant and buffy coat by aspirating with a 10-mL serological pipette.

5. Repeat **Section 3.3.5(2) through Section 3.3.5(3)** for a total of 3 washes, removing the supernatant each time.

6. Make a 0.5% RBC suspension by pipetting 500 µL of packed RBCs into 100 mL of PBS; mix by inverting.

7. Store at 4°± 2°C; use within a week of collection of the RBCs.

3.4 Preparation of the sample

3.4.1 On the day of the test initiation, thaw the Sample Transport Tubes containing the nasal swabs, express the fluid from the cotton by rotating and pressing the applicators against the tube wall, and vortex the media in the tubes to release virus from the swab. Discard the used cotton applicators, after expressing any fluid.

3.4.2 Centrifuge the sample at 500 x g (2000 rpm in a J6B centrifuge with a JS-4.0 rotor) for 20 ± 5 minutes. Transfer 2 mL of the supernatant from each sample into a new, labeled 12 x 75-mm polystyrene tube.

3.4.3 Keep the supernatant samples on ice until time of inoculation.

3.4.4 Store the remaining supernatant sample at -70°± 5°C.

4. Performance of the test

4.1 Label the Test Plate and aseptically decant the Growth Medium into a suitable container.

4.2 Inoculate 5 wells with 25 µL/well of each supernatant sample. Change tips between each sample.

4.3 Inoculate 5 wells/dilution with 25 µL/well of the PI_3V Positive Control (with dilutions 10^{-7} through 10^{-4} from the example in **Section 3.3.4[1]**). Tip changes are not necessary if pipetting from the most dilute (10^{-7}) to the most concentrated (10^{-4}).

4.4 Maintain 5 wells as uninoculated cell culture controls.

4.5 Incubate the Test Plate at 36°± 2°C in a $C0_2$ incubator for 60 ± 10 minutes for virus adsorption.

4.6 Add 200 µL/well of Maintenance Medium to the Test Plate with a multichannel pipettor.

4.7 Incubate the Test Plate at 36°± 2°C in a CO_2 incubator for 4 ± 1 days. Observe daily for PI_3V CPE which is characterized by cell fusion.

4.8 Harvest samples from each well in which typical PI_3V infection is observed into labeled 12 x 75-mm polystyrene tubes. Samples may be harvested at time of observation. Freeze at -70°± 5°C until next passage.

4.9 At the end of incubation, fluids from wells showing no CPE are passaged onto fresh Test Plates prepared according to **Section 3.3.3,** by repeating **Sections 4.2 through 4.7.** In addition, the harvested samples that displayed PI_3V infection are thawed and passed. A PI_3V Positive Control prepared as described in **Sections 3.3.4 and 4.3,** respectively, is included. At the end of the 4 ± 1 day incubation, an HAd test is performed on all second passage wells to confirm PI_3V infection.

4.10 HAd Test

4.10.1 Decant Maintenance Medium from the inoculated Test Plates (**Section 4.9**) in a suitable container.

4.10.2 Rinse all cells of the Test Plates by filling with PBS; decant.

4.10.3 Dispense 200 µL/well of a 0.5% of the RBC Suspension into all test wells.

4.10.4 Incubate the Test Plates 15 ± 5 minutes at room temperature (23°± 2°C).

4.10.5 Decant the RBC Suspension from the Test Plates and repeat **Section 4.10.2** for a total of 2 washes.

4.10.6 Examine the Test Plate monolayers on an inverted light microscope at 100X magnification, and record the results. Wells containing one or more RBC clusters adhering to the cell monolayer are considered to be positive for PI_3V.

4.11 Calculate the PI_3V endpoint of the PI_3V Positive Control using the method of Spearman-Kärber as commonly modified. The titers are expressed as $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$) of the test wells.

Example:

10^{-4} dilution of PI_3V Positive = 5/5 wells CPE/HAd positive
10^{-5} dilution of PI_3V Positive = 5/5 wells CPE/HAd positive
10^{-6} dilution of PI_3V Positive = 2/5 wells CPE/HAd positive
10^{-7} dilution of PI_3V Positive = 0/5 wells CPE/HAd positive

Titer = (X - d/2 + [d * S]) where:
X = $\log_{10}$ of lowest dilution (4)
d = $\log_{10}$ of dilution factor (1)
S = sum of proportion of CPE/HAd positive

$$\frac{(5 + 5 + 2)}{5} = \frac{12}{5} = 2.4$$

PI_3V Positive Control titer = (4 - 1/2 + (1 * 2.4) = 5.9

Titer of the PI_3V Positive Control is $10^{5.9}$ $TCID_{50}$/0.025 mL.

5. Interpretation of the test results

5.1 The uninoculated cell controls should not exhibit degradation or cloudy media that would indicate contamination.

5.2 The calculated HAd_{50} titer of the PI_3V Positive Control must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.3 Wells of Nasal Excretion Samples that tested HAd positive (regardless if CPE developed) are considered positive for PI_3V, whereas those wells of samples that did not develop CPE and tested HAd negative after the second serial passage in MDBK cells are considered to be negative for PI_3V.

5.4 Any Nasal Excretion Sample with 1 or more positive wells is considered to be positive for PI_3V**.** A Nasal Excretion Sample is only considered negative if all wells inoculated from the Nasal Excretion Sample are negative for PI_3V.

6. Report of test results

6.1 Results for each Nasal Excretion Sample are reported as positive or negative PI_3V isolation.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.309, U.S. Government Printing Office, Washington, D.C., 2007.

7.2 Cottral, G.E., (Ed.). *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca and London, 1978, pg.731.

7.3 Finney, D.J. *Statistical method in biological assay*. Griffin, London. 3rd ed., 1978, pg. 508.

8. Summary of revisions

This document was revised to clarify practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- Contacts, Joseph Hermann and Peg Patterson, have been added to the document.
- **1:** Primary bovine embryonic kidney cell cultures have been replaced by Madin-Darby bovine kidney-A cells.
- **2.2.3:** The description of the antibiotics added to the Diluent Medium has been changed to reflect the antibiotics used. Penicillin and streptomycin have been replaced by gentamicin sulfate, and Amphotericin B has been eliminated from the formula.
- **2.2.4:** Gentamicin sulfate has replaced penicillin and streptomycin for the transport medium.
- **2.2.13:** The 24-well cell culture plates have been replaced by 96-well plates.
- The term "PI_3V Reference" has been changed to "PI_3V Positive Control" throughout the document.